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Article

Defanging Chloroform – Mitigation of Deuterated Chloroform Decomposition to Stabilise Susceptible NMR Samples

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Abstract: Highly reactive decomposition products of (deuterated) chloroform can deteriorate samples dissolved in this commonly used NMR solvent. For sensitive samples (such as peptides, unsaturated fatty acids, vitamins), this can lead to abnormal NMR spectra (e.g. signal shifts depending on pH, attenuation of signals over time due to chemical changes of analytes, new signals from reaction products). Such irreproducibly influenced spectra are especially problematic for non-targeted analysis methods. To prevent these artefacts, chlorine, phosgene and hydrochloric acid need to be eliminated from deuterated chloroform prior to its use. Since the common stabilization methods have proven insufficient for sensitive NMR samples, another purging method has been tested: Mitigation is easily and reliably achieved by washing the deuterated chloroform with concentrated Na_2CO_3-solution and subsequent desiccation with oven-dried Na_2CO_3.

Keywords: NMR; chemometrics; chloroform; phosgene; hydrochloric acid; sample degradation; pH-shift; protonation

1. Introduction

Deuterated chloroform (chloroform-d) is a solvent widely used in NMR spectroscopy, due to its broad usability and comparably low price. On the other hand, it is well known that chloroform readily reacts with oxygen in a radical reaction, accelerated by light and yielding elemental chlorine, the highly toxic phosgene and hydrochloric acid [1–3]. In presence of water, phosgene is subsequently hydrolysed to carbon dioxide and hydrochloric acid (see F. 1 to F. 4). Even a low degree of chloroform-d decomposition will yield considerable amounts of aggressive reactants and lead to a pH-shift.

$$CHCl_3 + O_2 \xrightarrow{h\cdot\nu} Cl_3COOH \quad \text{(F. 1)}$$

$$Cl_3COOH \rightarrow \mathbf{Cl_2} + CO_2 + \mathbf{HCl} \quad \text{(F. 2)}$$

$$Cl_3COOH \rightarrow \mathbf{COCl_2} + \mathbf{HCl} + {}^1/_2\ O_2 \quad \text{(F. 3)}$$

$$COCl_2 + H_2O \rightarrow CO_2 + \mathbf{2\ HCl} \quad \text{(F. 4)}$$

This laboratory, as a part of the official food surveillance in the federal state of Baden-Württemberg, analyses a wide variety of samples, many containing unsaturated fatty acids or amino acids or vitamins. These substances are labile against acidity or degradation by chlorine, hydrochloric acid or phosgene, a powerful organic reagent [4,5]. This is especially problematic if the NMR spectra are intended for non-targeted analysis of samples, where high reproducibility of the data is a prerequisite for a reliable

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evaluation. Even slight changes in the sample solution can lead to shifts of signals in the spectra and can thus severely influence the chemometrical evaluation.

To quench the phosgene formation and therefore to stabilize the chloroform, usually a minor amount of ethanol, hydrocarbon or silver tape is added to chloroform. Ethanol is interfering in the radical chain reaction and converts phosgene to ethyl chloroformate [6]. In another way, chloroform is stabilized with traces of a hydrocarbon (e.g. cyclohexene, 2-penten, amylene). However, these act more as hydrochloric acid scavengers than as real stabilizers [6,7]. Since organic additives would lead to undesirable extra signals in NMR spectra, chloroform-d for NMR usage is often stabilized with silver tape as a halogen radical scavenger.

NMR spectra of samples in chloroform-d irregularly but repeatedly showed hints at sample deterioration and slight changes in pH value, despite of the usage of silver-stabilised chloroform-d stored in the dark. Even recently purchased, unopened bottles did not provide reliable assurance against deterioration and tainted spectra. Closer examinations let us presume chloroform deterioration is indeed the cause. An online research yielded only hints on how to quench chloroform with activated alumina or carbonate salts, but no detailed work instruction. Thus, the reliability of an easy method to purge chloroform-d of phosgene and hydrochloride was tried and tested.

The influence of "biting" chloroform on NMR samples was discussed at a recent German NMR conference, several laboratories had similar experiences, and thus a simple and reliable method preventing sample deterioration and bad spectra seems valuable.

2. Materials and Methods

Chemicals

Chloroform-d (99,8% deuterated, density approx. 1.5 g/mL, C. Roth, Karlsruhe, Germany); tetramethylsilane ("TMS", >99.9%, C. Roth, Karlsruhe, Germany). Sodium carbonate, anhydrous (K_2CO_3 could also be used, but phase separation will be hindered because its concentrated (>5 M) solutions have densities similar to that of chloroform-d.) (>99.9%, Sigma Aldrich (Merck), Darmstadt, Germany), (oven-dried > 24 h at 130 °C to 150 °C); sodium carbonate solution (aq.), 10 % m/v (~1 mol/L), density approx. 1.1 g/mL [8], aluminium oxide (activated, basic, Brockman I; Sigma Aldrich (Merck), Darmstadt, Germany)

Preparation

The initial volume of chloroform-d should allow for a loss of approx. 5% to 10%, this part is not efficiently recoverable after phase separation and drying. Plugs, sleeves for glass joints and seals should be made of PTFE, because chloroform attacks many other polymers.

Approx. 7 parts (v/v) of chloroform are mixed with 3 parts of the Na_2CO_3 (aq) solution and stirred or shaken vigorously for 5 to 10 minutes. The lower phase (chloroform-d) is separated for further processing; the aqueous upper phase should be disposed of as waste containing organic halogen.

To shake out small batches of chloroform-d, rather more carbonate solution is needed, ensuring a clearly visible phase boundary. Conical test tubes and a Pasteur pipette facilitate the handling of small batches.

The 10% carbonate solution ensures an excess to completely strip off traces of phosgene and hydrochloride. If there is concern that the chloroform-d is very old and potentially highly deteriorated (e.g. bubbling of CO_2 can be detected), the chloroform-d should be shaken out again with fresh carbonate solution.

Afterwards the chloroform-d is filled into a tightly closing brown glass bottle with a portion of oven-dried sodium carbonate. Approx. 50 g drying agent per litre chloroform-d have proven sufficient to bind residual water. For chloroform-d quantities of only a few millilitres, a slightly higher soda ratio is recommended, since a higher portion of tiny water drops cannot be separated beforehand.

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The filled and closed bottle should be preferably shaken, not stirred for two hours to enhance the removal of water residue. If a magnet bar stirrer is used, the soda could abrade PTFE particles into the liquid. The chloroform-d should be allowed to stand overnight to precipitate carbonate particles.

Immediately before usage, the "defanged" chloroform should be membrane filtered with a PTFE syringe filter to keep any particles out of the NMR sample.

3. Results

3.1. Example 1: Fat extracts of hens' egg yolks

The unwanted sample deterioration due to deteriorated chloroform-d was first observed when testing hen's eggs: To verify if samples come from organic production or not, a non-targeted method was developed by CVUA-KA's NMR laboratory [9]: Egg yolk is treated to yield a fatty extract, which is then measured by ^{1}H NMR. The spectrum is evaluated chemometrically and compared to a database of authentic samples (from both organic and conventional agriculture).

Intermittently the score plots of whole sample series were unexpectedly far "off model" (see Fig. 1), a check of the spectra revealed slight, but systematic shift changes of some signals (see Fig. 2).

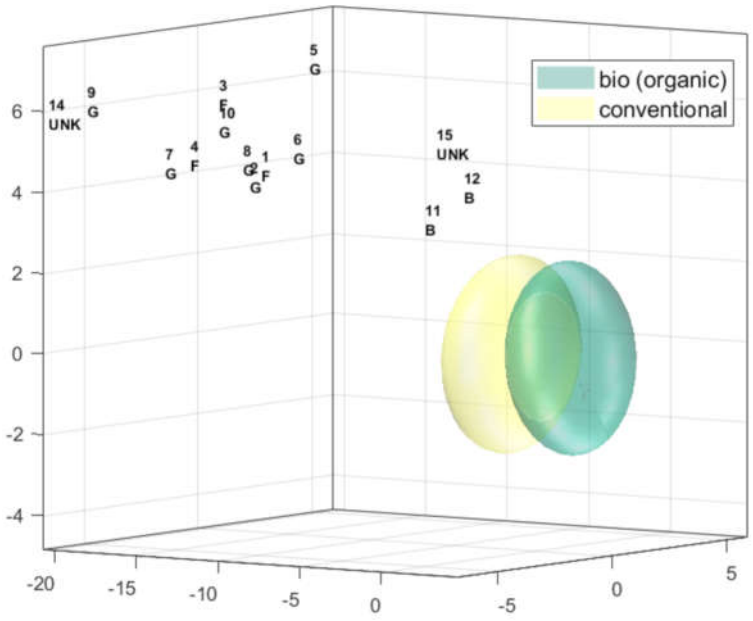

Figure 1. Score plot of egg samples (untreated chloroform-d was used) with very conspicuous sample scores having a very large offset. Sample scores labelled "B" (B for bio/ organic farming) should be in or close to the green right confidence ellipsoid, sample scores labelled "K-F" or "K-B" are expected close to or in the left, yellow cloud (K for conventional, F for free range, B for Boden, i.e. ground; unknown for undeclared keeping).

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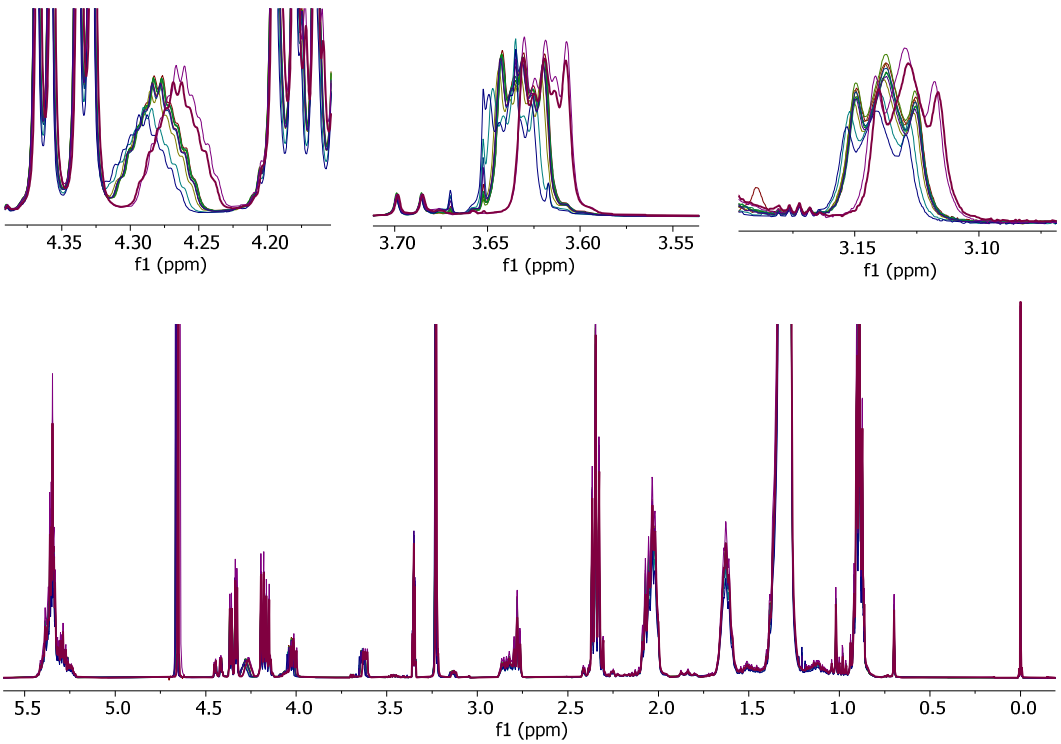

Figure 2. Superposition of thirteen ^{1}H-NMR spectra of fat extracts of egg yolk samples from different preparation batches. Solvent was untreated chloroform-d and MeOD-d4 (3/2, v/v). ^{1}H -NMR spectra, 400 MHz, 290 K, referenced to δ_{TMS} = 0.00 ppm. The zoomed sections (on top) show NMR resonances with inconsistent chemical shifts, presumably due to untreated chloroform. Under chemometrical evaluation, these signals will end up 3 to 10 bins away from their usual position due to the shift differences of ca. 0.01 to 0.03 ppm.

3.2. Example 2: Fat extracts of fish

The irregular NMR signal shifts due to untreated chloroform-d, which were observed in spectra of hen´s egg samples, raised the question whether other food matrices were also affected. As mentioned before, unsaturated fatty acids are sensitive to degradation by chlorine radicals. Since fish fat contains a high ratio of unsaturated fatty acids, it was suspected that this matrix might also be affected. Furthermore, residual water and degradation products of untreated chloroform-d could lead to a general signal shift of polar metabolites in the ^{1}H NMR spectrum of fish fat samples. These shifts can lead to degraded separation or even incorrect conclusions in subsequent multivariate data analysis of the samples. Especially in the range from δ_{H} = 8.0 – 8.7 ppm, unwanted signal shifts occurred due to the usage of untreated chloroform-d (Fig. 3A). By using the defanged chloroform-d, these could be significantly reduced to shift variances ≤ 0.01 ppm of the respective signal (Fig. 3B).

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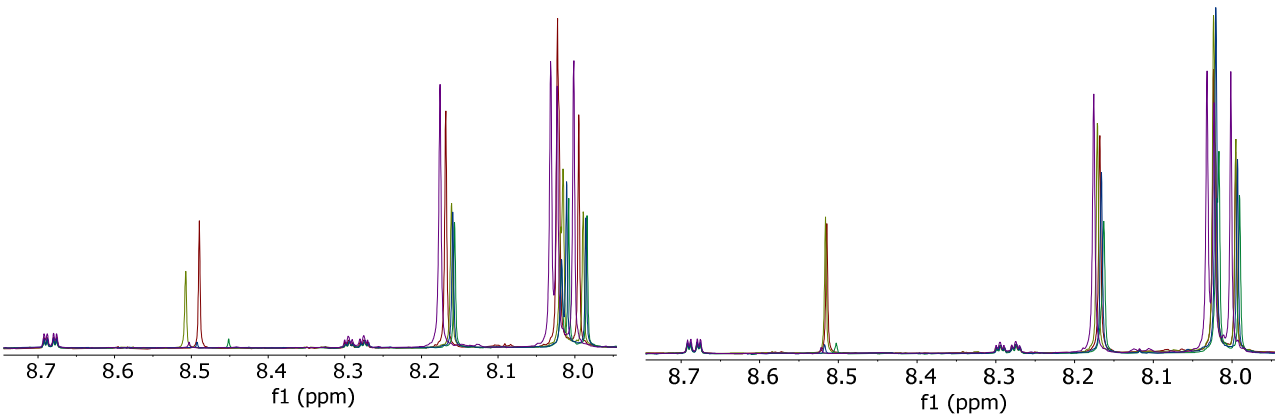
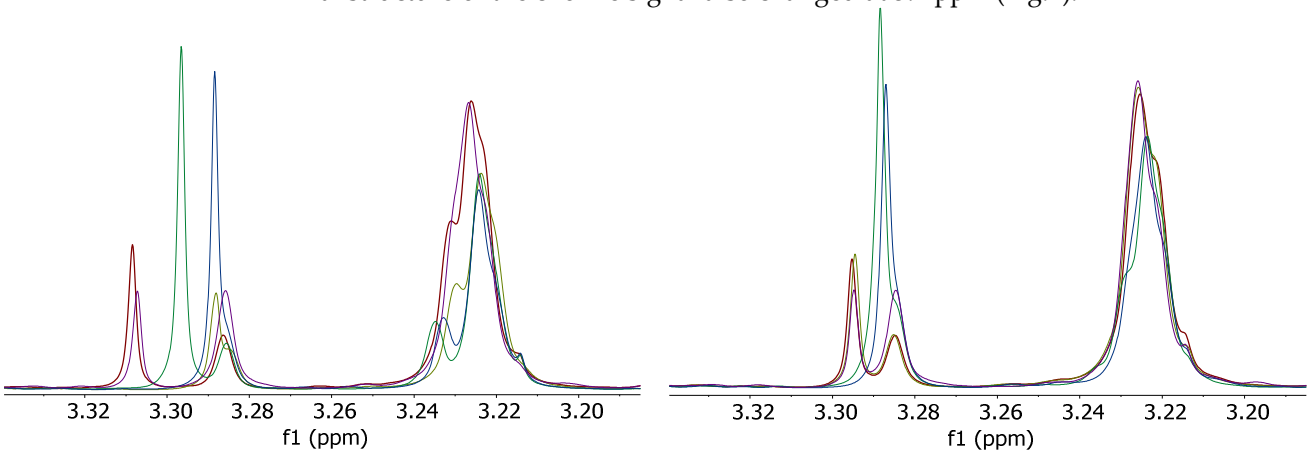


(**a**) (**b**)

Figure 3. Superposition of five spectra in the range of δH = 7.95 – 8.75 ppm of fat extracts from trout samples. (**a**) Solvent was **untreated** chloroform-d and MeOD-d4 (2/1, v/v); (**b**) **"defanged"** chloroform-d and MeOD-d4 (2/1, v/v). (^{1}H-NMR spectra, 400 MHz, 290 K, referenced to δ_{TMS} = 0.00 ppm).

Furthermore, when untreated chloroform-d was used, signal shifts were detected in the spectral region where the signals of the phospholipids appear, (Fig. 4A). Similar to the signals in the previously described high-ppm region of the spectrum, these shift variances could be reduced by the application of defanged chloroform-d (Fig. 4B). Besides, the signal structure of the choline signal also changed at 3.2 ppm (Fig.4).



(**a**) (**b**)

Figure 4. Superposition of five spectra in the range of δ_H = 3.21 – 3.33 ppm of fat extracts from trout samples. **(a)** Solvent was **untreated** chloroform-d and MeOD-d_4 (2/1, v/v) and **(a) "defanged"** chloroform-d and MeOD-d_4 (2/1, v/v). (^{1}H-NMR spectra, 400 MHz, 290 K, referenced to δ_{TMS} = 0.00 ppm.).

3.3. Example 3: Coffee extracts

Undesirable signal shifts were also observed in NMR spectra of coffee fat extract. Here, for multivariate data analysis, the lipophilic metabolites of coffee were extracted and measured by ^{1}H-NMR. The irregular signal shifts occurred predominantly in the range of δ_H = 8.0 – 9.2 ppm (Fig 5A) and could be reduced by the application of defanged chloroform-*d* (Fig 5B). Using chloroform-*d* treated by the method described above, shifted NMR signals were no longer observed.

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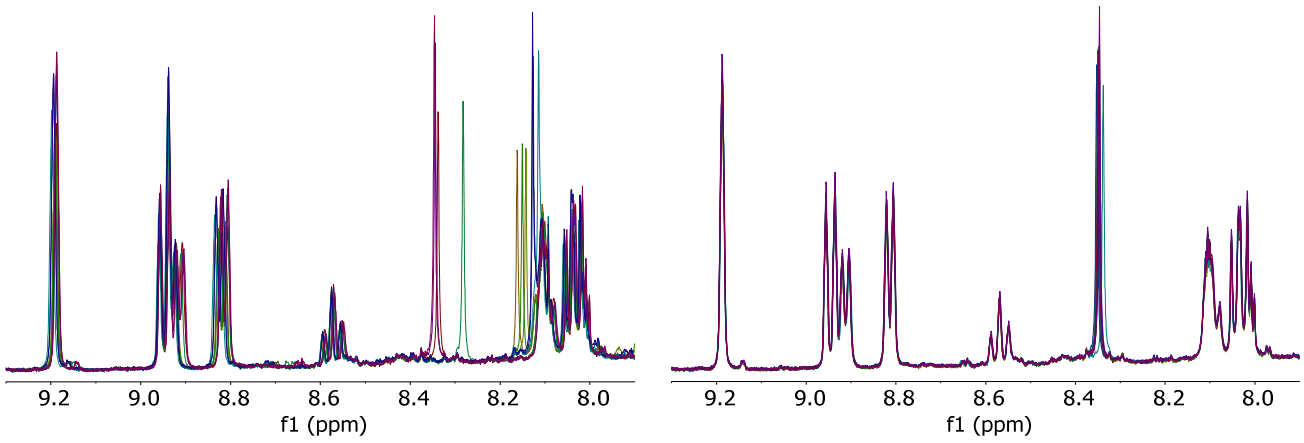

(**a**) (**b**)

Figure 5. Superposition of ten spectra in the range of δ_H = 7.9 – 9.3 ppm of a fat extract of the same coffee sample. **(a)** Solvent was **untreated** chloroform-d and MeOD-d4 (1/1, v/v)) and **(a) "defanged"** chloroform-d and MeOD-d_4 (1/1, v/v). (^{1}H-NMR spectra, 400 MHz, 290 K, referenced to δ_{TMS} = 0.00 ppm.).

3.4. Removal of residual water

Two sets of NMR samples were prepared, the first using defanged chloroform-d just desiccated for one hour by shaking with oven-dried sodium carbonate, the second using defanged chloroform-d desiccated more thoroughly by shaking with oven-dried sodium carbonate for 2 hours and then a rest period overnight before sample preparation.

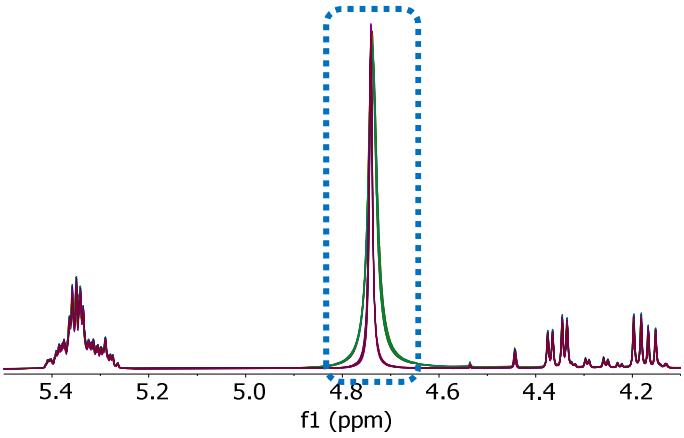

Figure 6. Superposition of 24 spectra in the range of δH = 4.1 – 5.5 ppm of fat extracts of the same coffee sample. The water signal is highlighted in blue. Solvent was defanged chloroform-d and MeOH-d4 (1/1, v/v). For one set (a dozen spectra, green) the defanged chloroform-d was shaken for one hour with oven-dried sodium carbonate (green spectra), then used immediately. For the other set (a dozen spectra, maroon) the chloroform-d was shaken for several hours with oven-dried sodium carbonate and allowed to stand overnight (purple spectra). (^{1}H-NMR spectra, 400 MHz, 290 K, referenced to δTMS = 0.00 ppm.).

As shown in Fig. 6, one hour of shaking with oven-dried sodium carbonate is not sufficient to remove residual water. The procedure A more thorough desiccation, as described in the methods above, can further reduce the residual water resulting in improved 1H NMR spectra in a smaller and narrower with only weak water signals in the 1H NMR spectrum.

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3.4. Alumina as an alternative mitigating agent

For a quick comparison with another possible option of mitigating chloroform, basic alumina was obtained and a portion was added to untreated chloroform-d (ratio: 5g per 100 mL), then the mixture (slurry) was vortexed for ten minutes. Afterwards, the alumina was separated by filtration and the clean, treated chloroform-d used to prepare exemplary samples of egg fat, olive oil and coffee extracts for NMR analysis. Each group of replicate spectra was very well aligned, no relevant signal shifts could be found, but the treated chloroform had a much higher $CHCl_3$-Signal compared to the untreated chloroform. Probably Alumina seems to speed up the D-H-exchange of $CDCl_3$, not unexpected, considering the relative acidity of chloroform and alumina's alkalinity.

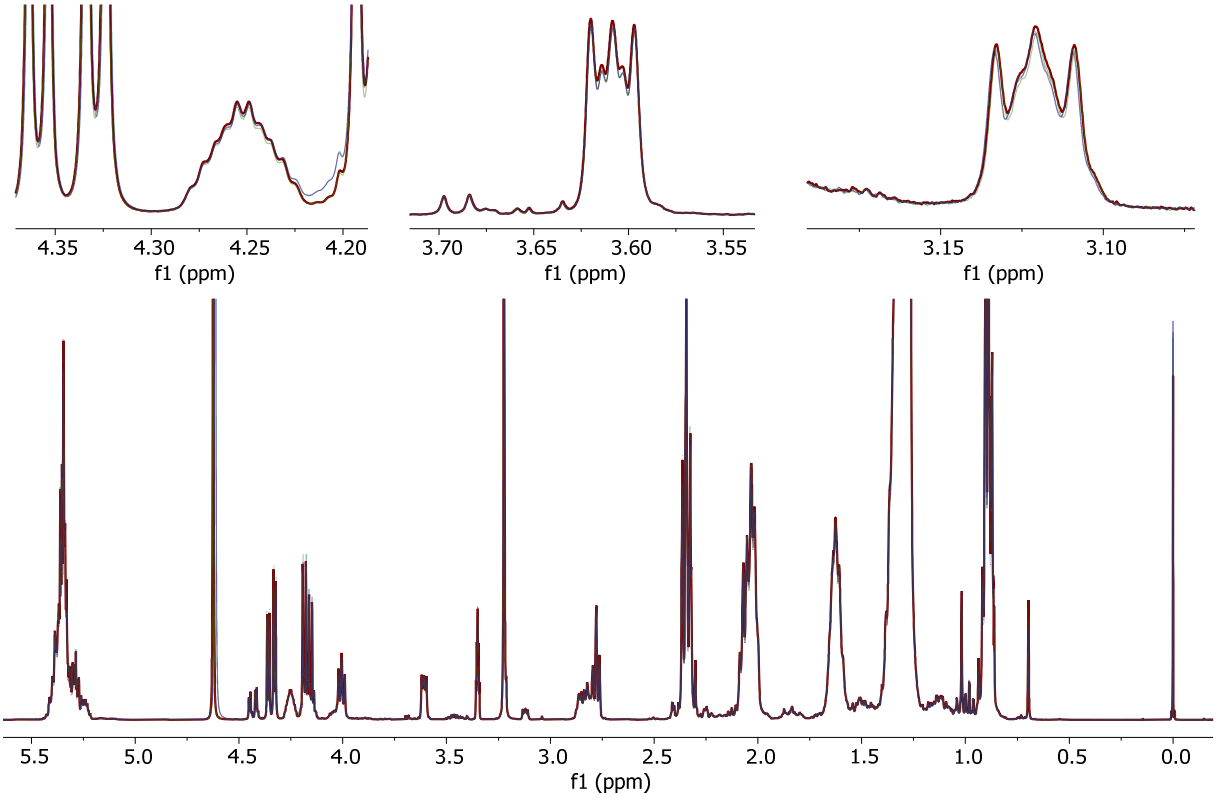

Figure 6. Superposition of four 1H-NMR spectra of fat extracts of egg yolk samples. Solvent was chloroform-d (treated with alumina) and MeOD-d4 (3/2, v/v). (^{1}H-NMR spectra, 400 MHz, 290 K, referenced to δTMS = 0.00 ppm.)
Graphic layout is similar to Fig. 1; no strange chemical shifts are visible. Under chemometrical evaluation, no erroneous binning is to be expected and further evaluation of the data will not be hindered.

4. Discussion

As described in the results of example 1 to 4, signal shifts and changes in signal shape were observed when non-treated chloroform-d for the NMR measurement was used. To the best of our knowledge, there is no literature, which describes NMR signal shifts and changes in signals shape because of decomposition products in chloroform-d. As described before, chloroform can be stabilized in several ways with ethanol being the most efficient way [10–12]. However, the usual stabilising agents for chloroform have certain disadvantages if the chloroform is intended as an NMR solvent: They lead to additional unwanted signals in the 1H NMR spectra (ethanol, amylene) or do not remove all of the aggressive decomposition products (silver foil). As described by Fuhrmann et al., Moody et al. and Verpoorte et al. [11,13,14] decomposition products in chloroform can react with components containing a piperazine-ring, phospholipid fatty acids or nitrogen containing compounds. This led to a loss of analyte, artefact formation and formation of undesired

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reaction products. The reaction of the decomposition products of chloroform-d with the sample might be the reason of differences in the signal shape of choline in the fat extracts of fish or the appearance of new signals can occur.

Furthermore, Tsujikawa et al. measured an acidic pH value of insufficiently stabilized chloroform [10]. This could probably lead to the irregular 1H-NMR signal shifts of the polar metabolites in the examples above.

The simple procedure presented has proven so far to reliably remove the problem of degraded or pH-shifted (protonated) samples due to chlorine, phosgene or hydrochloride in chloroform-d. As the chloroform is stored over sodium carbonate, the removal of phosgene and hydrochloride is ensured for at least a week, presumably much longer. A reshaking may be recommendable after longer storage at rest.

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